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博 士 学 位 论 文

海洋石油降解菌及烷烃单加氧酶(AlkB 与 AIMA) 基因多样性分析与食烷菌烷烃降解基因的研究

Diversity of Marine Oil-Degrading Bacteria and their alkane monosxygenase genes (AlkB and AIMA) and Alkane Degradation Genes of Alcanivorax Bacteria

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摘要

石油是海洋环境中的重要污染物。微生物的降解作用对于清除溢油和环境自净非常重要,石油污染环境的生物修复技术成功应用取决于对海洋环境中石油降解菌多样性及其降解机制的深入了解。

厦门港是重要的国际码头。本论文通过对厦门近海环境样品进行柴油、石油的分别富集培养,分离筛选得到 44 株参与烃降解的细菌,这些菌的 16S rRNA 基因系统发育分析表明,分属于变形菌门、放线菌门、拟杆菌门、厚壁菌门的细菌,表明该区域的石油降解微生物种类较丰富。共分为 26 个属,其中有 4 个可能的新属、6 个可能的新种。单菌验证结果表明,其中 28 株有烷烃降解能力,15 株降解效果明显。PCR-DGGE 分析表明,在所有富集菌群中优势降解菌为 *Alcanivorax*、*Acinetobacter* 和 *Alcaligenes*。利用简并引物 *alkBwf* / *alkBwr*,对上述的 44 株可培养单菌进行了 *alkB* 基因的扩增,从 10 个属的 15 株细菌里扩增共获得 16 个 *alkB* 基因序列,并首次从 *Gallaecimonas*、*Castellaniella*、*Paracoccus* 和 *Leucobacter* 4 个属发现 *alkB* 基因。此外,还以烷烃富集菌群 DNA 为模版,进行了 *alkB* 基因的扩增,共获得了 45 个 *alkB* 基因序列,其中 29 个不同于来自可培养菌株的 *alkB*。系统进化分析表明,这些 *alkB* 基因大致可分为 5 类同以报道的 *alkB* 基因有 60-80% 同源性。荧光定量 PCR 分析表明,每升表层海水中细菌 16S rRNA 基因总量为 $(1.02 \pm 0.13) \times 10^9$ 至 $(1.66 \pm 0.22) \times 10^9$ 个拷贝,各类 *alkB* 基因大约有 3×10^3 至 3×10^5 个拷贝。

在大西洋烷烃降解菌的 *alkB* 基因多样性调查方面,本文利用简并引物 *alkBwf* / *alkBwr*,对分离自大西洋表层海水的 197 株可培养单菌进行了 *alkB* 基因的扩增,从 11 个属的 47 株细菌里获得了 52 个 *alkB* 基因序列。其中大部分 *alkB* 基因大部分来自于变形菌门的 α 、 β 和 γ 类群,少部分来自于放线菌门以及厚壁菌门,包括 *Alcanivorax*、*Bacillus*、*Brachybacterium*、*Gordonia*、*Kordiimmonas*、*Leifsonia*、*Marinobacter*、*Microbacterium*、*Salinisphaera*、*Tetrathiobacter* 等。在这些菌中,有 7 个属的细菌为首次报道,分别为 *Bacillus*、*Brachybacterium*、*Gordonia*、*Kordiimmonas*、*Leifsonia*、*Microbacterium* 和 *Tetrathiobacter*。系统进化分析表明,这些 *alkB* 基因多种多样,大致将其分为 5 类,并且主要来自各种食烷菌。

AlmA 是新发现的长链烷烃氧化酶^[108], 多样性及底物范围研究较少。为分析 *almA* 基因多样性, 本文首先设计了简并引物 AlmAdf/AlmAdr, 通过条件优化的 PCR 反应程序, 从分离自石油富集菌群的单菌和菌群 DNA 中共获得了 102 个 *almA* 基因序列。利用可培养菌株, 从 4 个食烷菌模式种中得到了 6 个 *almA* 基因序列; 在 137 株南海可培养细菌中, 有 3 个属 26 株菌中共获得了 28 个 *almA* 基因序列; 从分离自印度洋和太平洋表层海水的 28 株细菌中, 有 5 株细菌共获得了 8 个 *almA* 基因序列。这些 *almA* 基因主要来自变形菌门的 α 和 γ 亚门, 最常见的属包括 *Alcanivorax*、*Bacillus*、*Marinobacter* 和 *Salinisphaera*。此外, 从对厦门和大西洋表层海水富集菌群中分别获得了 26 和 34 个 *almA* 基因序列。系统进化分析表明, 获得的 *almA* 基因多种多样, 并且主要来自各种 *Alcanivorax* 和 *Marinobacter*。

Alcanivorax dieselolei 重要的海洋石油降解菌, 前期工作中我们从该种的模式菌株B-5中发现了2个*alkB*基因和1个*P450*基因, 并进行了功能初步验证。本文又从中发现了1个*almA*基因, 与已报道的有烷烃降解功能的单加氧酶同源性较低。将上述这些基因分别在*P. putida* GPo12 (pGEC47 Δ B) 和突变株*P. fluorescens* KOB2D1中进行异源表达后结果表明, *alkB*, *P450*和*almA*基因分别编码一个有功能的烷烃单加氧酶。通过mini-Tn5转座突变, 获得了*almA*基因的突变株。该突变株与野生型菌株相比, 在长直链烷烃和支链烷烃生长明显下降。为了进一步证明*almA*基因的功能, 通过*E. coli*异源表达、酶活的测定, 发现纯化的AlmA蛋白有烷烃单加氧酶活性。烷烃诱导Q-PCR定量分析表明, *P450*的底物范围主要为C₈-C₁₆; *AlkB*的底物范围主要为C₁₂-C₂₆; *AlmA*的底物范围主要为C₂₂-C₃₆; *alkB1*和*almA*基因分别能被支链烷烃 (Pristane和 Phytane) 诱导表达。可见, 菌株B-5的三套烷烃单加氧酶系统的底物范围有互补性。这种多基因的策略有助于该菌能够有效地利用多种烷烃生长而具有较宽的底物范围。

Alcanivorax hongdengensis A-11-3是本课题组发现并鉴定的另一株高效烷烃降解的食烷菌新种。本研究从该菌株中也发现了三套烷烃单加氧酶系统, 包括 2个*alkB*基因、3个*P450*基因 和2个*almA*基因。通过基因组DNA的Fosmid文库构建, 分别获得了菌株A-11-3的2个*alkB*基因和3个*P450*基因的基因簇。为了进一步验证这些基因的功能, 分别将其在*P. putida* GPo12 (pGEC47 Δ B) 和突变株

P. fluorescens KOB2D1中异源表达。结果表明, 这些*alkB*和*P450*在菌株A-11-3中分别编码了一个有功能的烷烃羟化酶。RT-PCR分析表明, *alkB*和*P450*的底物范围为短链或中链长的直链烷烃, *alkB1*和*P450-3*基因还能被支链烷烃(Pristane)诱导表达; *almA1*基因被长链烷烃(C₂₈-C₃₂)的诱导表达, 而*almA2*基因中能在更宽范围的长链烷烃(C₂₄-C₃₄)和支链烷烃诱导下上调表达, 但两者均不受22碳以下烷烃(C₉-C₂₂)的诱导。此外, 分析发现在*alkB*和*P450*的上游或下游总有一个调节基因存在, 这些调节基因分别是*TetR*、*GntR*、*Ara/xyls*、*AraC*和*MscS*。

总之, 本文通过厦门近海、大西洋的表层海水样品中的石油降解菌群及*alkB*基因和*almA*基因多样性进行了系统的研究, 获得了丰富的石油烃降解菌, 发现了多种多样的烷烃降解基因。此外, 本研究中对*A. dieselolei* 和*A. hongdengensis*的烷烃单加氧酶作进一步研究, 初步揭示其利用烷烃的分子机制。这些结果对于我们了解石油在海洋环境中的归宿提供了参考, 也为进一步开发石油污染生物修复制剂提供了宝贵的材料。

关键词: 海洋石油污染; 石油降解菌; 烷烃羟化酶; 食烷菌; 黄素结合单加氧酶 (AlmA)

Abstract

Nowadays, natural oil seepage, marine oil transport accidents lead to persistent and serious pollution on marine environments and ecosystems. Bioremediation by virtue of microbial biodegradation is now widely recognized as an effective tool to remove marine oil pollution. It has long been recognized that many microorganisms which are abundant and widespread in environments have evolved to use this highly reduced alkanes as a diet. Thus, the isolation and identification of oil-degrading bacteria and study of their mechanisms are very important and necessary on bioremediation of oil spills in both basic research and applied research.

In this report, the diversity of oil-degrading bacteria and *alkB* gene was surveyed in the seawater around Xiamen Island. Consequently, a total of 44 bacterial isolates consisting of 26 genera were obtained, which were characterized by 16S rRNA analysis. They belonged to *Proteobacteria*, *Actinobacteria*, the CFB group and *Firmicutes*, among which the α - and γ -*Proteobacteria* consisted of the majority. Besides, at least 6 potential novel species were isolated. Most of the obtained isolates exhibited growth with diesel oil and crude oil. PCR-DGGE analysis revealed that, the cultivable bacteria belonging to the following genera of *Alcanivorax*, *Acinetobacter* and *Alcaligenes* dominated these oil-degrading consortia. *alkB* genes were positively detected in 16 isolates by degenerate PCR. And for the first time, *alkB* genes were found in bacteria of *Gallaecimonas*, *Castellaniella*, *Paracoccus* and *Leucobacter*. Additional 29 *alkB* sequences were retrieved from genomic DNA of the oil-degrading communities. Phylogenetic analysis showed that the obtained *alkB* genes formed 5 groups, most of which exhibited 60-80% similarity at the amino acid level with sequences retrieved from the GenBank database. Further, the abundance of *alkB* genes and 16SrRNA genes in seawater were examined by real-time PCR, respectively. The results showed that *alkB* genes of each group *in situ* ranged from about 3×10^3 to 3×10^5 copies L^{-1} , with the homologs of *Alcanivorax* and *Pseudomonas* being the most predominant; total bacteria 16S rRNA gene copy number ranged

from $1.02 \times 10^9 (\pm 0.13 \times 10^9)$ to $1.66 \times 10^9 (\pm 0.22 \times 10^9)$ copies L^{-1} . Bacteria of *Alcanivorax*, *Acinetobacter* and *Pseudomonas* are important oil-degraders in this area; while those frequently reported in other area, like *Oleiphilus* spp., *Oleispira* spp. and *Thalassolituus* spp. were not found in our report. These results indicate that bacteria and genes involved in oil-degradation are quite diverse, and may have restriction in geographic distribution in some species.

A total of 197 bacterial isolates consisting of 48 genera were obtained, which were characterized by 16S rRNA analysis from surface seawater across the Atlantic Ocean [64]. Using newly designed degenerate PCR primers, *alkB* genes were surveyed in these 197 culturable marine bacteria. Together, 52 *alkB* gene fragments were obtained from 47 bacterial strains, which belonged to 11 genera, such as *Alcanivorax*, *Bacillus*, *Brachy bacterium*, *Gordonia*, *Kordiimmonas*, *Leifsonia*, *Marinobacter*, *Microbacterium*, *Salinisphaera*, and *Tetrathiobacter*. Among them, the following genera were reported for the first time to harbor *alkB* genes: *Bacillus*, *Brachy bacterium*, *Gordonia*, *Kordiimmonas*, *Leifsonia*, *Marinobacter*, *Microbacterium*, *Tetrathiobacter* and *Solimonas*. Phylogenetic analysis showed that this kind of genes were quite diverse and formed several clusters, most of which were generated from various *Alcanivorax* bacteria. Interestingly, some sequences were grouped into a far related novel cluster, such as those from *Salinisphaera* genus. On the other hand, far related isolates owned similar or even identical sequences, indicating horizontal gene transfer.

Alcanivorax dieselolei strain B-5 is a marine bacterium that can utilize a broad range of *n*-alkanes (C_5 - C_{36}) as sole carbon sources. However, the mechanisms responsible for these characteristics remain to be established. In this report, the characterization of four alkane hydroxylases was surveyed from *A. dieselolei*, including two homologues of AlkB (AlkB1 and AlkB2); a CYP153 homologue (P450), as well as an AlmaA-like (AlmA) alkane hydroxylase. Heterologous expression of *alkB1*, *alkB2*, *p450*, and *almA* verified their functions in alkane oxidation. The *A. dieselolei* B-5 *almA* mutant MAB5 did not grow with C_{32} and C_{36} alkanes as a sole carbon source, and did short

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